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Effects of Photobioreactor Depth on *Stichococcus* Cultures Aimed at Biodiesel Production

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Biofuels production by microalgae is a challenging process known since the end of the last century. Bio-oil extracted from microalgae may be adopted as crude fuels or may be transesterified to biodiesel. Key aspects for industrialization of microalgae-to-biofuel processes are: selection and improvements of algal strains; development of high-performance photobioreactor; improvement of oil extraction and transesterification. The present contribution regards the effects of reactor design on biodiesel production by *Stichococcus bacillaris* of *Naegeli genus.* The attention was focused on the effects of the depth of sub-horizontal flat photobioreactors on the performances of continuous cultures. Two rectangular shape columns were investigated of depth 5 and 8 cm.
Semi-continuous conditions were investigated as regards the biomass suspension. Tests were carried out at 23°C and pH 7. The continuous irradiance level was set at 240 $\mu E\ m^{-2}s^{-1}$, gas flow rate at 0.5 vvm, CO_2 concentration in the gas stream set at 2%v. Cultures were characterized in terms of pH, concentration of total nitrogen, total organic carbon, total inorganic carbon, biomass, lipids fraction and methyl-ester distribution of transesterified lipids.
Results pointed out that the performance of the photobioreator decreased with the photobioreactor depth: the specific productivity referred to the irradiated surface increased when the light path decreased.

1. Introduction

The 20th century has been characterized by explosive growth in energy consumption and rapid increase in greenhouse effects. The exploitation of alternative/renewable energy sources is one of the most relevant priorities of countries overburdened with energy bills heavily dependent on fossil fuels. Main issues of concerns are:

- the world's fossil fuel reserves are limited;
- the fossil fuel reserves are unevenly distributed in the world;
- flue gases from combustion of fossil fuels cause the greenhouse gas effect and the consequent warming of earth's atmosphere.

Global warming has been mainly attributed to the increase of CO_2 level in the atmosphere (Etheridge et al., 1998). The critic threshold of 450 ppm CO_2 has been exceeded 10 years before the predicted data and several solutions have been investigated to reduce/control the CO_2 emissions (Wang et al., 2011; Fortună et al., 2012; Coppola et al., 2012; Russo et al., 2013). Particular challenging results the CO_2 capture coupled to bioenergy production, the use of biological materials for energy purposes. Although the high potential of these processes, their development at industrial scale is still waiting for economical successful solutions (Marzocchella et al., 2010; Stephens et al., 2010). Photosynthetic organisms - plants, algae, and some photosynthetic bacteria - efficiently utilize the solar energy to convert water and CO_2 – preferably from air or exhausted gas streams - into renewable energy vectors (biomass). Microalgae - as higher plants – convert the solar energy in chemical energy stored as triacylglycerols (TAGs). TAGs could be

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used to produce of a wide variety of chemicals and they may be converted in fatty acid methyl esters (FAMEs) to be used as transport fuels (Chisti, 2007; Posten and Schaub; 2009, Francisco et al., 2010). FAMEs – or better known as biodiesel - can be synthesized from TAGs through a transesterification reaction (Sheehan et al., 1998).

The biofuel production coupled with the CO_2 sequestration by means of photosynthetic microorganisms has been considered as a promising process since the end of the last century (Benemann et al., 1977). Chisti (2007) reported that biodiesel production rate from microalgae cultures may be 1–3 orders of magnitude larger than that from oil crops. In fact, microalgae grow rapidly and many strains are exceedingly rich in oil (50–80%$_W$). Moreover, the microalgal biomass fixes a large amount of CO_2 – 1.83kg of CO_2 per kg of dry microalgae – and strongly contributes to the reduction of greenhouse gas emissions. Pollio et al. (1997) indicated *Stichococcus bacillaris* (ACUF 158/11) as a potential candidate for fuel production because of the high tolerance to large variations of temperature, salinity, and pH, and of its lipid content (33%). In previous works Olivieri et al. (2011, 2012, 2013) reported about tests carried in vertical bubble columns and in inclined bubble columns photobioreators – operated with different CO_2 concentration in the gas phase and pH of the medium - on biodiesel production by *S. bacillaris*. Cultures carried out in inclined bubble columns resulted characterized by biomass and oil productivity (256 and 80 mg/L d, respectively) significantly higher than those assessed in vertical bubble column (124 and 42 mg/L d).

Present contribution moves one step further along the direction of investigating the effects of photobioreactor depth on biodiesel production by *S. bacillaris*. Tests were carried out in inclined bubble columns characterized by 5 and 8 cm depth. Semi-continuous conditions were adopted. Cultures were characterized in terms of pH, concentration of total nitrogen (TN), total organic carbon (TOC), total inorganic carbon (IC), biomass (X_{ss}), lipid fraction (%) and methyl-ester distribution of transesterified lipids.

2. Experimental

2.1 Organism and medium

Stichococcus bacillaris was from the ACUF collection of the Department of Biological Science at the University of Studies of Napoli “Federico II” (http://www.biologiavegetale.unina.it/acuf.html). Bold Basal Medium (BBM) supplemented with $NaNO_3$ (40 mg L^{-1}) as nitrogen source was adopted (Bold 1949, Bischoff and Bold 1963). BBM was autoclaved for 20 minutes. The final pH was about 6.8.

2.2 Reactor setup

Erlenmeyer flasks housed in a climatic chamber (Gibertini) were adopted for microalgae pre-cultures. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55).

Bubble column photobioreactors (Figure 1) were prism with rectangular cross section. The longitudinal axis of the photobioreactors was inclined by 20° with respect to the horizontal plane. Two designs were adopted: 2 L column with 8 cm of depth, and 1 L column with 5 cm of depth. Gas stream was sparged at the bottom of the photobioreactors by means of multiple-orifice (1 mm ID) Teflon tube. A hydrophobic filter (0.2 μm) sterilized the gas flow inlet. A gas mixing device (M2M engineering) provided the selected concentration of CO_2 in the gas stream fed to the photobioreactors by mixing air and pure carbon dioxide from a pressurized vessel.

The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations. The photobioreactors where housed in climate chamber (Heraeus Vötsch GmbH - HPS 500) equipped with lamps at the ceiling

2.3 Diagnostics

Bacterial and fungal contamination in the reactors was checked by microscope observations with a magnification factor of 40 X and 100 X, respectively (Leitz Wetzler; 567146; Germany). The pH was measured with a pH-meter (Crison, Basic 20). The concentration of algal biomass (X) was estimated as optical density at 600 nm with a spectrophotometer (Specord 50 – Analytic Jena). A fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs) was used to measure the content of in vivo chlorophyll a (Chl A) in the untreated samples.

Photosynthesis and oxygen uptake rate were off-line measured by means of a Hansatech Oxygraph adopting 1 mL untreated samples. The analysis of the TOC, TN and IC was carried out with a TOC-V CSH total organic carbon analyzer of SHIMADZU (Model: TNM-1).

Harvested microalgae by centrifugation for 10 min at 5000 rpm and 4 °C, were processed to characterize the lipid and biodiesel composition according to the following steps: I) freeze-drying at -50°C (Labconco

Freezon); II) lipid extraction with a 2:1 chloroform-methanol solvent mixture in a Soxhlet apparatus for 8 h; III) transesterification with methanol and 1.5 %w NaOH at 65 °C for 3 min; IV) methyl esters analysis through HPLC (Agilent 1100) (mobile phase: water (90%) and acetonitrile (10%) column: Synergi 4u; detector: UV/Vis, 210 nm). The identification and quantification of the fatty acid methyl esters (FAMEs) by chromatographic peaks was performed using commercial standards from Sigma-Aldrich

2.4 Operation conditions and procedure

The working volume of bubble columns was set at 1.7 and 0.6 L, respectively, for the thick (8 cm depth) and the thin (5 cm depth) configuration. The volumetric flow rate of the sparged gas stream was set at 0.5 vvm in both photobioreactors. The temperature of the climate chamber was set at 23±1 °C. Tests were carried out under continuous irradiance set at 240 μE m^{-2} s^{-1}. CO_2 concentration in the sparged gas was set at 2%.

Pre-cultures were carried out in the 200 mL Erlenmeyer flasks - containing 100 mL BBM medium - under continuous irradiance set at 50 μE m^{-2} s^{-1} (Olivieri et al., 2011). Subcultures were carried out transferring 50 mL of 4-5 day cultures into the Erlemeyer flasks supplemented with 50 mL of fresh BBM. 3-4 week cultures were inoculated into photobioreactors adopting a dilution ratio typically of 1/10.

Operation of bubble column photobioreactors included (Olivieri et al., 2011): i) start-up under batch conditions for two weeks; ii) fed-batch conditions for one month; iii) semi-continuous conditions for two months. Under fed-batch conditions a concentrated BBM (10 times the concentration of pre-cultures) was supplemented to the photobioreactors when TN was lower than 0.01 g/L. The volume of concentrated BBM was 1/10 of initial culture volume and it almost balanced the liquid losses by sampling and water evaporation. Under semi-continuous operations 35% of microalgal suspension was weekly replaced with fresh medium. Therefore the average dilution rate (D) was assessed as the ratio between the weekly-replaced suspension volume and the photobioreactor working volume (V), accordingly it resulted 0.05 d^{-1}. Semi-continuous operations were typically extended for at least six weeks. Then, the photobioreactors were operated for a week under nitrogen starvation conditions.

Steady states were characterized in terms of biomass concentration (X_{ss}), lipid concentration, and chlorophyll a concentration whose values were calculated as the average over the last month of culture. Biomass productivity ($W_X = X_{ss} \bullet D$) was assessed for each test. The biomass superficial productivity (A_X), at steady state conditions, was assessed as the product of W_X and the photobioreactor depth.

3. Results and discussion

Figures 2 reports data regarding two tests carried out in inclined bubble column photobioreactors of 8 cm of depth and 5 cm of depth, respectively. The tests refer to cultures carried out in pH 7 broth sparged with

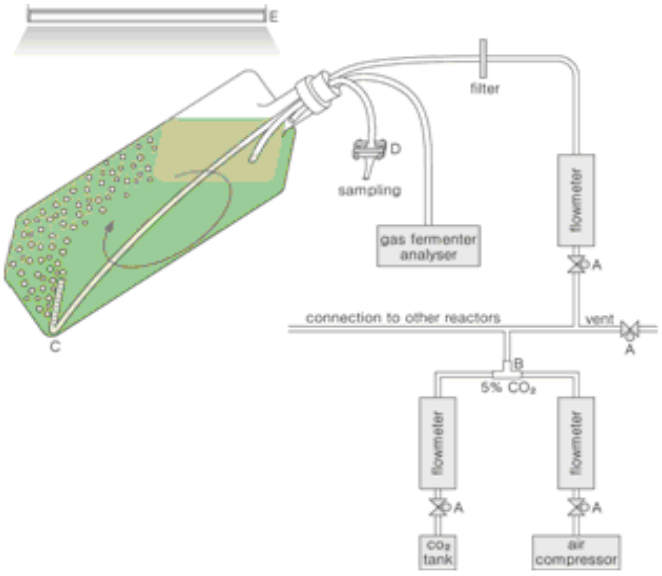

Figure 1: Sketch of the inclined square bubble column photobioreactor (not on scale): A) valve, B) gas mixer, C) gas diffuser, D) sterile clamp, E) light source.

air enriched with CO_2 at concentration of 2%. Batch conditions were adopted for three weeks. As the concentration of nutrients in the medium was not sufficient (t=18 day) the fed-batch mode started. The biomass concentration increased during the fed-batch mode and it approached a maximum value of about 4.5 g L^{-1} in both photobioreactors. At t=32 day the semi-continuous mode started with a weekly replacement of 35% of the suspension corresponding to a dilution rate of 0.05 d^{-1}. Provided that a steady state condition was established, the reactors were operated under semi-continuous mode for about 6 weeks. During this time interval, the replaced broth was collected to assess lipids produced under nitrogen sufficient conditions. At t=75 day the broth was replaced with a medium without the nitrogen source so the nitrogen starvation conditions started. The cultures were carried out for one week under nitrogen starvation conditions. At about 12 weeks the cultures were stopped and the residual biomass were collected in order to assess the lipids produced under nitrogen starvation.

Table 1 reports the results of tests in terms of concentration of biomass, chlorophyll a content, biomass productivity (expressed on both volumetric and exposed area bases) assessed for each steady state. The composition of identified methyl esters are also reported in Table 1 for the steady state conditions and for the nitrogen starvation conditions.

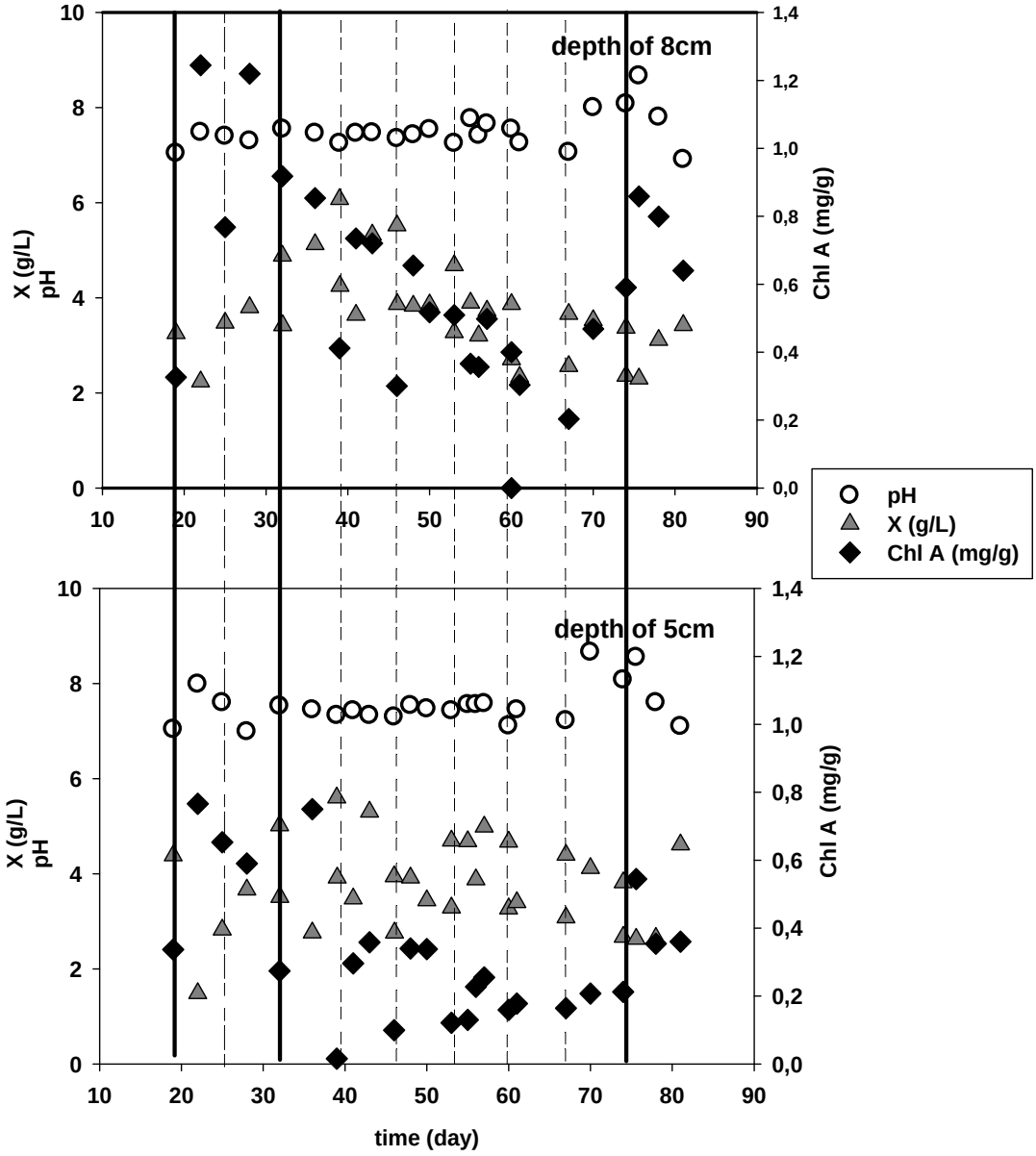

Figure 2: Stichococcus bacillaris cultures. Continuous lines mark the beginning of the fed-batch, semi-continuous and nitrogen starvation conditions. Dashed lines mark the weekly broth/medium substitution.

Table 1: Steady state data of semi-continuous tests in inclined bubble column photobioreactors under N sufficient (+) and N starvation (-) conditions.

Photobioreactor	**5 cm of depth**				**8 cm of depth**			
CO_2 - %	2				2			
pH	7				7			
X_{ss} – g L^{-1}	4.00		4.99		5.13		5.12	
W_X – g L^{-1} d^{-1}	0.20		0.25		0.25		0.25	
A_X – g m^{-2} d^{-1}	0.011		0.014		0.023		0.023	
Chl A –mg g^{-1}	0.46		0.49		0.23		0.24	
N conditions	+	-	+	-	+	-	+	-
Esterif. lipid - % ([a])	6.01	6.06	4.99	7.06	5.84	9.79	5.57	12.02
C18:1cis–oleate, %([b])	0.30	0.81	0.31	1.16	0.58	1.77	0.47	1.55
C18:1-elaidate, %([b])	0.42	0.89	0.30	0.55	0.62	1.86	0.51	2.25
C18:2 – linoleate, %([b])	3.72	2.93	3.79	3.73	3.27	5.66	3.67	5.25
C18:3 - linolenate, %([b])	1.58	1.43	0.59	1.63	1.37	0.49	0.92	2.96

([a]) Data referred to total lipids
([b]) Data referred to total esterified lipids

The biomass reached steady state conditions in about four weeks from the beginning of the semi-continuous regime in both photobioreactors. It is interesting to note that under steady state conditions, the biomass concentration (X_{ss}) and the biomass volumetric productivity (W_X) were about constant with the photobioreactor depth: about 4.8 g L^{-1} and 0.24 g L^{-1} d^{-1}, respectively. On the contrary, chlorophyll a content and biomass superficial productivity changed with the biomass depth. The chlorophyll a content decreased with the photobioreactor depth from 0.48 mg g^{-1} (5 cm depth) to 0.23 mg g^{-1} (8 cm depth). It is interesting to note that under steady state conditions, the biomass concentration (X_{ss}) and the biomass volumetric productivity (W_X) were about constant with the photobioreactor depth: about 4.8 g L^{-1} and 0.24 g L^{-1} d^{-1}, respectively. Accordingly, the biomass superficial productivity (A_X) increased with the photobioreactor depth from 0.0125 g m^{-1} d^{-1} (5 cm depth) to 0.023 g m^{-1} d^{-1} (8 cm depth).
Results indicate that maximum lipid percentage was achieved in the culture operated in the thick photobioreactors under nitrogen starvation: 12.2% $_w$. No difference was observed in terms of percentage of transesterified lipids under nitrogen sufficient conditions.
As regard the lipid composition methyl-oleate was the highest identified chemical in the biodiesel.
The effects of the photobioreactor depth deserve some considerations. The microalgae productivity was about constant with the depth and the chlorophyll content decreased with the depth. The first issue suggests that the reaction volume of the inclined column photobioreactor was exploited with the same efficiency for both depths. However, the microalgal concentration was sufficiently high to affirm that the light decay rapidly within the photobioreactor and just a few mm layer close to the exposed surface should be under sufficient irradiated source. It may infer that the frequency of microalgae turnover at the irradiated surface was sufficient to expose microalgae at the light source at sufficient frequency and period. It is known that chlorophyll *a* content of numerous algae is inversely proportional to the light intensity during growth (Stewart, 1974) Algal physiology and biochemistry, ed. W.D.P. Stewart, 1974, University of California Press). The strong reduction of chlorophyll *a* content observed in 8cm-depth phobioreactors could be due to the more frequent exposition of algal cells to the light source.

4.Final remarks

The effects of the light path on photobioreactor performances was successful investigated. Tests were carryed out in rectangular shaped columns characterized by different depth – 5 and 8 cm - and inclined with respect to the vertical axis.
The growth of *Stichococcus bacillaris* was affected by the the photobioreactors depth under operating conditions tested. The biomass productivity was about constant (0.24 g L^{-1} d^{-1}) with depth if volume unit was adopted as reference, it increased with the photobioreactor depth - 0.0125 g m^{-1} d^{-1} for 5 cm depth and 0.023 g m^{-1} d^{-1} for 8 cm depth - if irradiated unit surface was adopted as reference.

The invariance of the biomass volumetric productivity rate with the photobioreactor depth – under operating conditions tested - may be interpreted taking into account the microalgae turnover at the irradiated surface.

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